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Distinct Enzymatic Responses in Mice Exposed to a Range of Low Doses of Ozone

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Short-term exposure of mice to low O_3 doses, as defined by the product of concentration and exposure time (*ct*), was observed to induce alterations in two enzyme systems: first, that leading to changes in hepatic reduced ascorbic acid (RAA) content, and second to changes in plasma creatine phosphokinase (CPK) activity. RAA alterations were noticed immediately, 30 min and 120 min after termination of the exposure period, whereas CPK showed alterations immediately and 15 min after termination of the exposure. Later determinations, i.e., 24 hr after O_3 exposure for RAA and 30 min after 0_3 exposure for CPK, revealed no significant differences when compared to control animals. Although differences in sensitivity existed, the dose response curves for both systems were more or less similar, showing a short decrease for the initial very low O_3 doses, followed by a profound rise and a gradual decrease to control levels for subsequent ct doses. Exceptions were the 30 min curve for RAA and the immediate curve for CPK in so far as that both showed an additional depression.

Neither plasma histamine nor plasma lactic acid dehydrogenase (LDH_3) were observed to be altered by the range of O_3 doses employed. These findings were explained on the basis of adaptation of the organism to a potentially noxious O_3 stimulus by enhanced metabolic processes: a weak stimulus leading to only a small adjustment, and stronger stimuli to elevated enzyme activity as well. With increasing doses of O_3 this elevation in enzyme activity was found to be gradually diminished, possibly due to a steadily growing demand, leaving the overshoot becoming continually smaller until a balanced state is achieved.

Radiomimetic activity of ozone (O_3), first described by Brinkman and Lamberst (*1*) and supported by the suggestion of Goldstein et al. (*2-4*) that O_3 action in living organisms may be mediated by the formation of free radicals, might have led investigators to consider every O_3 action as being harmful regardless of the O_3 concentration and the duration of exposure (*5*, *6*). In the opinion of these authors there is no threshold value (no-effect level), and the dose-effect curve starts at zero. However, in the same way as with ionizing irradiation, in order to visualize effects, dosages of O_3 have to be explored which in general clearly exceed the permissible value for industrial workers (i.e., 0.1 ppm/8 hr = 200 $\mu g/m^3$/8 hr).

Exposure times of days or weeks have often been used under experimental conditions. There is no doubt that, in the higher concentration time ranges, O_3 causes toxic symptoms. The linearity of the dose-effect relationship of hazardous O_3 action, down to zero, must be disputed, as it has been for ionizing irradiation (*7*). Even more so, since nonanthropogenic O_3 is a natural ambient constituent for which an adaptive mechanism to lower values might be supposed to be present. Currently, such a mechanism has actually been suggested in the results of a number of studies, including those done in man (*8-11*).

The results of the present study, where mice are exposed to O_3 doses in a range regularly occurring in ambient air, are in favor of the existence of such an adaptive process.

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Materials and Methods

Groups of 15-20 male mice of an inbred grey strain (Gron), as well as of the hybrid of this strain with inbred C_{57}bl mice (F_1) were exposed to O_3, with five mice to a cage. Similar groups of mice were concurrently sham-exposed. Cages were placed in a stainless steel cabinet. The animals had full access to water, but they were deprived of food for 16 hr prior to and in the period of O_3 exposure. O_3 was generated by an adjustable silent-discharge ozonizer (Air-cos Switzerland) situated at the bottom of the cabinet.

The oxidant was monitored by a Dasibi type 1003AH ozonometer (Dasibi, USA) operating on the principle of UV absorption by O_3. Blood for investigation was drawn from the orbital sinus under light ether anaesthesia.

Reduced ascorbic acid (RAA) in the liver was determined as previously described (*12*). Creatine phosphokinase (CPK) in blood plasma was assayed with the CPK test-combinations of Boehringer-Mannheim (W. Germany). Plasma histamine was determined according to the single-isotope method described by Beaven et al. (*13*), with some minor modifications. In our study, the sensitivity of this method was approximately 1 ng/ml. Total lactic acid dehydrogenase (LDH) in blood plasma was assessed with the LDH test-combinations of Boehringer-Mannheim (W. Germany). LDH isoenzymes were separated by disc gel electrophoresis (*14*), and the gels quantitatively scanned by means of a Gilford model 2400 S automatic spectrophotometer with linear transport equipment operating at 500 nm (Gilford, Ohio, USA). Results were expressed in percentages of those found in the controls and plotted against the product of concentration (c) and exposure time (t). As has been demonstrated earlier, when c and t are adapted so that the product ct remains constant, identical results are obtained (*15*). Student's t-test and a test based on normal approximation were used for statistical evaluation of the results. The level of significance was set at 5%. The standard error of the percentage difference between experimental and control values at each ct value was calculated as 100 times the square root of the expression:

$$\frac{(SE_2)^2}{(\bar{X}_1)^2} + \frac{(SE_1)^2\,(\bar{X}_2)^2}{(\bar{X}_1{}^2)^2}$$

where SE_1 = standard error of the mean of control values, SE_2 = standard error of the mean of experimental values, $\bar{X}_1$ = mean of control data, and $\bar{X}_2$ = mean of experimental data.

Results

The data obtained for hepatic RAA for three different post-exposure time intervals, i.e., for 0, 30, and 120 min, are plotted in Figure 1. Each of the three curves starts with a short negative period, followed by a sharp rise to values significantly above zero. Thereafter the curves level off to normal at the higher ct values tested. A few measurements performed 24 hr after O_3 exposure reveal RAA values falling within the normal range.

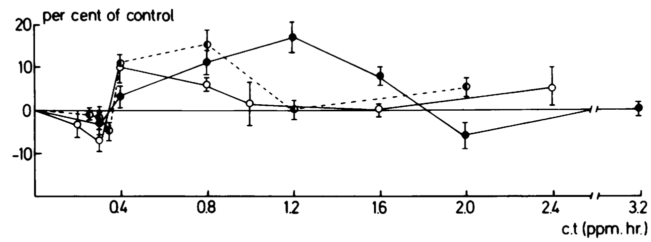

FIGURE 1. Reduced ascorbic acid (RAA) content of murine liver tissue expressed as a percentage of control values at three different time intervals after exposure of the animals to various concentrations of ozone (O_3) for various time periods (ct): (○) immediately after exposure; (●) 30 min after exposure; (O) 120 min after exposure. Each point is an estimate based on the results from at least 40 experimental animals and 40 controls. The vertical bars at each point represent the standard error of this estimate. Maximal O_3 concentration 1600 $\mu g/m^3$; maximal exposure time 4 hr.

Similar curves were obtained for plasma CPK immediately and 15 min after O_3 exposure (Fig. 2). this enzyme appears to be more sensitive, in that lower ct values are capable of evoking alterations in the enzyme activity. Furthermore, the effect of O_3 on CPK disappears more rapidly; 30 min after termination of the O_3 supply, significant alterations were no longer observed. Values obtained 15 min after O_3 exposure also failed to show significant changes, with the exception of the CPK value obtained at ct = 0.8.

The histamine content of blood plasma remained unaltered after treatment of mice with a series of O_3 concentrations for various time periods (Table 1). In addition, both total LDH as well as its isoenzyme remaining within normal limits (Table 2).

Discussion

Since RAA is a product of a chain of enzymatic activities, and since O_3-induced changes in RAA are of an enzymatic nature (*12*), we have in fact

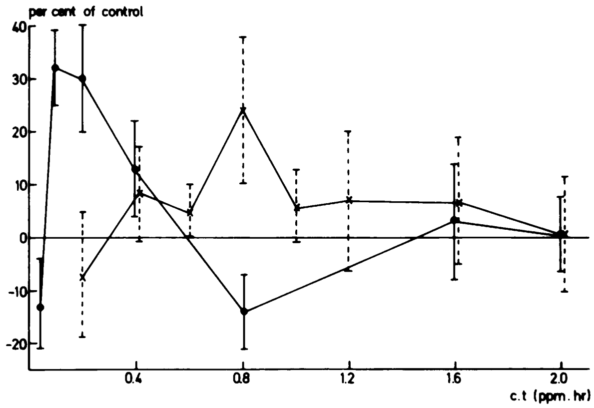

FIGURE 2. Creatine phosphokinase (CPK) activity of murine blood plasma expressed as a percentage of control values at two different time intervals after exposure of the animals to various concentrations of ozone (O_3) for various time periods (*ct*): (●) immediately after exposure; (×) 15 min after exposure. Each point is an estimate based on the results from at least 30 experimental animals and 30 controls. The vertical bars at each point represent the standard error of this estimate. Maximal O_3 concentration 1600 $\mu g/m^3$; maximal exposure time 4 hr.

recorded alterations in two enzymatic systems in this study.

Although differences in sensitivity between the two systems occur, the alterations are principally similar. They are restricted to the lower O_3 doses tested. This, together with the rapid reversibility of the effects may indicate that we are dealing with a physiological phenomenon rather than with cellular injury. In this sense, the initial negative part of the curves obtained might just represent a certain metabolic adjustment due to weak O_3 action. Subsequent higher doses may stimulate enzyme activity (an all-or-none effect) (*12*) in concert with increasing consumption until a new equilibrium may be achieved where experimental and control values are similar. Such a course with increasing O_3 doses might be characterized as adaptive and noninjurious. This is supported by the finding that plasma histamine and LDH_3 are not significantly changed. A decrease in histamine has been demonstrated in the lungs of mice and rats exposed to oxidants (*16-18*), whereas increased levels of plasma LDH_3 are suggested to be indicative of lung tissue damage (*19*).

The use of *ct* values seems justified under the conditions employed in this study, where low O_3 concentrations and short exposure times are combined. The method has been validated for toxicological research (*20*) and has also been used in air-pollutant investigations (*21*). We have also verified this method for some enzymes using a single *ct* product with different *c* and *t* values and the product was found to be constant (*15, 22*).

The sensitivity of different enzyme systems in reacting to an adverse stimulus appears to be different. At least, lower *ct* doses are capable of inducing alterations in plasma CPK compared with those leading to modified RAA values of the liver.

Other enzymes have been observed to show altered activity after O_3 exposure, for example, acetylcholinesterase (Ach-ase) activity is decreased in the erythrocytes of mice after exposure to 8 ppm O_3 for 4 hr (*3*).

Increases in rat lung glutathione peroxidase (GSHP), glutathione reductase (GSHR) and glucose-6-phosphate dehydrogenase (G-6-PDH) have been observed by Chow and colleagues (*5, 23-25*) after several doses of O_3, the lowest dose being 4 ppm for 8 hr.

G-6-PDH as well as LDH are enhanced, whereas Ach-ase is lowered in human erythrocytes after exposure to 0.5 ppm O_3 for 2.75 hr. Serum GSHR is concomitantly decreased (*8*).

Plasma glutamate pyruvate transaminase (SGPT) rises in mice treated with 0.2 ppm O_3 for 2 hr (*12*).

Ishiwatari (*26*) has also found an increase in pulmonary GSHP in rabbits and mice exposed to at least 5 ppm O_3 for 4 hr. Glucose-6-phosphatase is concurrently lowered in murine lungs, whereas

Table 1. Mean plasma values of histamine determined either immediately or 2 hr after exposure of mice to various concentrations of O_3 compared to simultaneously determined values of nonexposed controls.

Exposure		Mean plasma histamine ± SEM, $\mu g/m^3$ (no. of animals)			
		Immediately		After 2 hr	
Time, hr	O_3 concn, $\mu g/m^3$	Controls	Exposed	Controls	Exposed
2	100			11.2 ± 1.5 (9)	11.6 ± 1.5 (11)
2	140	15.0 ± 1.9 (21)	19.0 ± 2.1 (20)		
2	400	8.6 ± 0.6 (10)	9.1 ± 0.9 (12)	13.9 ± 1.0 (37)	14.5 ± 1.0 (44)
2	1600	24.6 ± 1.4 (16)	23.1 ± 2.7 (23)	21.0 ± 2.9 (11)	18.6 ± 2.0 (13)

Table 2. Mean plasma values of total LDH and LDH_3 determined either immediately or 15 min after exposure of mice to various concentrations of O_3 for various time periods as compared to simultaneously determined values for nonexposured controls.

Exposure			Mean plasma values of enzyme ± SEM, U/l. (no. of animals)			
			LDH		LDH_3	
Time, hr	O_3 concn, $\mu g/m^3$	Time of determination	Controls	Exposed	Controls	Exposed
1	800	Immediate	212 ± 7 (9)	226 ± 8 (9)	24 ± 1 (9)	25 ± 1 (9)
2	400		215 ± 9 (14)	235 ± 8 (13)	26 ± 2 (14)	24 ± 2 (13)
4	2000		227 ± 14 (20)	240 ± 12 (18)	26 ± 2 (20)	31 ± 2 (18)
2	800	After 15 min	249 ± 16 (13)	211 ± 14 (12)	—	—
2	1200		277 ± 32 (14)	274 ± 21 (17)	40 ± 3 (14)	49 ± 4 (17)

ATP-ase shows lower values in the lungs of rabbits exposed to 10 ppm O_3 for 2 hr per day on five successive days.

Lee et al. (*27*) have treated rats with 0.2, 0.5, and 0.8 ppm O_3 for 1 to 30 days and obtained elevated levels of lung succinate oxidase, cytochrome-c reductase and also G-6-PDH.

In man the values for Ach-ase, G-6-PDH and phosphokinase in red blood cells are unaltered after exposure to 0.2 ppm during a normal working day, but LDH and α-hydroxybutyrate dehydrogenase levels decrease (*25*).

In most cases cited above, the O_3 doses used were significantly higher than those for which we observed altered enzyme activity, but the reversibility of the reaction has not been considered. Although elevated blood levels of some enzymes are indicative of tissue injury, and most authors tend to explain their findings to be those of an injurious nature, one may question whether the possibility of adaptation has been sufficiently taken into account, as was done by Buckley et al. (*8*) and Hackney et al. (*9*).

The second depression in two of our curves, at ct = 0.8 and at ct = 2.0 for CPK, determined immediately, and for RAA 30 min after O_3 exposure respectively, cannot be precisely interpreted. They certainly point to metabolic imbalance. In both cases later values (at 15 min and 2 hr after exposure respectively) show a positive overshoot.

Increased RAA levels have been claimed (*12*) to contribute to the organism's defense. CPK is involved in the generation of ATP from creatinephosphate, and in this way supports the easily available energy source of the organism. This might also be considered to be part of the defensive potency of the organism. In fact, mobilization of defense may imply a scala of diverse reactions which will stabilize at a certain level if the noxious stimulus is not too strong. At that point the organism reaches the adaptive state.

We explored O_3 doses equal to or slightly higher than those occurring in environmental air. To conclude that these O_3 doses can be safely borne is premature, since we tested only one animal species. Moreover, O_3 is frequently present in combination with other pollutants and our knowledge of combined effects is fragmentary (*12*, *29-31*). The occurrence of mutational events under influence of air pollutants is still under discussion (*32*, *33*).

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